AUGMENTATION OF SPONTANEOUS
MACROPHAGE-MEDIATED CYTOLYSIS
BY EOSINOPHIL PEROXIDASE*

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Perturbation of the plasma membrane of the macrophage by certain pharmacologic, immunologic, or particulate stimuli provokes a burst of extramitochondrial respiration, leading to the secretion of partially reduced forms of molecular oxygen, such as superoxide anion (1) and hydrogen peroxide (2). These reactive oxygen intermediates can lyse tumor cells within a few hours after stimulation of the activated macrophage respiratory burst (3–6).

With more prolonged incubation, activated macrophages sometimes lyse tumor cells spontaneously, that is, in the absence of agents known to trigger the respiratory burst (7). Several oxygen-independent mechanisms of spontaneous macrophage-mediated cytotoxicity have been described (8–11). It is less clear whether oxygen-dependent mechanisms can also play a role (12–16). In fact, there is little evidence that macrophages secrete consequential amounts of reactive oxygen intermediates in the absence of exogenous triggering agents (1, 2).

To investigate this issue, we made use of the unique peroxidase (EPO)[1] contained in the granules of eosinophils (17). This enzyme, which is distinct from myeloperoxidase, enhances the destruction of fungi (18), bacteria (19–21), schistosomula (22), toxoplasma (23), trypanosoma,[2] mast cells (24), and tumor cells (25) in the presence of exogenous H_2O_2 and halide. EPO is highly cationic (21) and can adsorb to microbes such as *Staphylococcus aureus* (26), *Toxoplasma gondii* (23), and *Trypanosoma cruzi*,[2] markedly potentiating their killing during phagocytosis by mononuclear phagocytes. We asked whether EPO adsorbed to tumor cells would enhance spontaneous cytotoxicity by activated macrophages.

Materials and Methods

Purification of EPO. EPO was purified from horse eosinophils as previously described (21). Briefly, eosinophils were isolated in 98–100% purity from 10–40 liters of horse blood, the EPO

[1] *Abbreviations used in this paper:* BCG, bacillus Calmette-Guérin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DMSO, dimethylsulfoxide; EPO, eosinophil peroxidase; E/T ratio, ratio of peritoneal cells to tumor cells; GO, glucose oxidase; MEM-(0.25, 5, or 10%)HS, Eagle's minimum essential medium, alpha variant, with 100 μg/ml streptomycin, 100 U/ml penicillin, and the indicated percentage of heat-inactivated horse serum; PMA, phorbol myristate acetate.

[2] Nogueira, N. M., S. J. Klebanoff, and Z. A. Cohn. 1982 *T. cruzi*: sensitization to macrophage killing by eosinophil peroxidase. *J. Immunol.* In press.

extracted with 0.05 M sodium acetate buffer, pH 4.7, containing 0.18 M sodium chloride, and the enzyme purified by chromatography on Sephadex G-200 and carboxymethylcellulose. The final product, which was present in 0.05 M sodium acetate buffer, pH 4.7, with 1.0 M sodium chloride, had a 415/280 nm optical density ratio of >0.9. Peroxidase activity was determined by guaiacol oxidation (27). 1 U of enzyme is the amount that oxidizes 1 μmol of guaiacol/min at 25°C, using a molar absorbancy for the product, tetraguaiacol, of 2.66×10^4 cm^{-1} at 470 nm (28). The activity of freshly prepared stock solutions ranged from 112 to 340 U/ml. Aliquots (10–50 μl) of the EPO preparation were frozen in microhematocrit tubes and thawed just before use.

Treatment of Tumor Cells with EPO. Tumors were carried as ascites in the mouse strains indicated: LSTRA lymphoma (BALB/c), TLX9 and NK lymphomas (C57BL/6), and P815 mastocytoma [(BALB/c × DBA/2)F$_1$ (CD2F$_1$)]. TLX9 and P815 were also maintained for up to 3 wk in stationary suspension culture in Eagle's minimum essential medium (alpha variant) with 100 μg/ml streptomycin and 100 U/ml penicillin, containing 10% horse serum heated at 56°C for 30 min (MEM-10% HS). Tumor cells were labeled with Na$_2$^{51}CrO$_4$ as previously described (ref. 29 for LSTRA; ref. 30 for TLX9, NK, and P815). For coating of tumor cells with EPO, 15×10^6 LSTRA cells in 0.5 ml of Hanks' balanced salt solution containing 0.001% gelatin and 10 mM glucose, or 1×10^6–3×10^6 TLX9, NK, or P815 cells in 0.5 ml of Krebs-Ringer buffer containing 6 mM phosphate and 5.5 mM glucose, were incubated with 10–20 μl of the EPO preparation for 10–15 min at 37°C. The dilution of the stock EPO preparation was >25 times, so that the final acetate buffer and sodium chloride concentrations contributed by the enzyme preparations were <0.002 M and 0.04 M, respectively. The cells were washed twice and suspended in 0.1 M phosphate buffer pH 7.0 (for LSTRA), MEM-5% HS (for TLX9 and P815), or MEM-0.25% HS (for NK lymphoma). Control (uncoated) tumor cells were treated identically, except that EPO was not added. The cells were counted and viability assessed with 0.2% trypan blue in a hemocytometer.

Effector Cells. Peritoneal cells were collected from untreated or variously injected CD2F$_1$ female mice, washed, enumerated, and counted differentially as described in detail elsewhere (2, 3, 5). The cells were suspended at 3×10^5/0.1 ml of MEM-5% HS (for use with TLX9 or P815 cells) or MEM-0.25% HS (for use with NK lymphoma cells), except where noted otherwise. Where indicated, 7×10^6 peritoneal cells in 2 ml Krebs-Ringer buffer were layered on top of 3 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) in a 15-ml tube, and centrifuged at 300 g for 10 min at 4°C. The upper, mononuclear cell layer was washed three times in the medium used for the assay.

Cytolysis Assay. For studies performed in the absence of effector cells, uncoated and EPO-coated target cells were incubated with the components of the reaction mixture as described in the legends to Tables and Figures. With LSTRA, the reaction was conducted in 10 × 75-mm polystyrene test tubes (2054; Falcon Labware, Oxnard, CA) and ^{51}Cr release determined as previously described (29). With the other tumor cells, microtest plates were used as described below.

For studies using effector cells and target cells, 0.1 ml of each were dispensed to round-bottomed microtest plates (Flow Laboratories, Rockville, MD) containing 0.02 ml of phorbol myristate acetate (PMA) diluted in dimethylsulfoxide (DMSO) or DMSO alone (final concentration, 0.033% DMSO with or without 100 ng/ml PMA). Where indicated, catalase (from beef liver, type C100, Sigma Chemical Co., St. Louis, MO) or sodium azide (Fisher Scientific Co., Pittsburgh, PA) were diluted in normal saline and added in a volume of 0.02 ml to the assay wells. Catalase was inactivated by boiling for 20 min, reconstituting to the original volume with distilled water, and sonicating 15–30 s (power output 1, Heat Systems-Ultrasonics Inc., Plainview, NY) to disperse the precipitate. The plates were incubated at 37°C in 5% CO$_2$/ 95% air for 4.5 h and centrifuged. Gamma emissions were counted in 0.1 ml of each supernatant. Percent specific lysis for triplicate cultures was calculated as $(A - B)/(C - B)$, where A equals percent release of ^{51}Cr in the presence of effector cells, B equals percent release in the absence of effectors, and C equals percent of the total label that was releasable in the presence of 0.2% Triton X-100 (Sigma Chemical Co.). The simple proportional SEM for triplicates was calculated as before (3). In this paper, "spontaneous release" refers to term B as defined above,

whereas "spontaneous lysis" refers to specific lysis in the presence of effector cells but in the absence of PMA.

Percent Inhibition. Inhibition of EPO-dependent cytolysis by catalase or azide was expressed as $100 \, (D - E + F)/D$, where D equals specific lysis of EPO-coated tumor cells in the absence of catalase or azide, E equals specific lysis of EPO-coated targets in the presence of either agent, and F equals specific lysis of targets not coated with EPO. The value of F averaged 8.2% and was the same whether or not catalase or azide was present. In computing E, the term for spontaneous release pertained to tumor cells incubated with the same concentration of catalase or azide but without effector cells. No concentration of either agent as used here significantly affected the spontaneous release of ^{51}Cr.

Other Reagents and Procedures. 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) was from Bristol Laboratories, Syracuse, NY. Where indicated, protamine sulfate (preservative free; Eli Lilly and Co., Indianapolis, IN) was used in place of EPO at a concentration of 0.06 mg/ml. Glucose oxidase (GO) from *Aspergillus niger* (Type V) was from Sigma Chemical Co. The rate of production of H_2O_2 was assayed by the scopoletin method (2). All other details of procedure, and the sources of mice and materials, were as previously reported (3, 5, 30).

Results

Increased Sensitivity of EPO-coated Tumor Cells to Lysis by H_2O_2. In an earlier study (25), EPO from guinea pig peritoneal cells sensitized LSTRA lymphoma cells to lysis by 10^{-4} M preformed H_2O_2 in the presence of a halide. We confirmed this finding with a highly purified preparation of horse EPO, and in addition determined the effect of allowing the EPO to bind to the surface of the tumor cells by preincubation. Table I demonstrates the toxic effect of H_2O_2 and a halide on LSTRA lymphoma cells with surface-bound EPO. Each component of the toxic system (EPO, H_2O_2, and halide) was required. The hemeprotein inhibitors azide, cyanide, and aminotriazole decreased the toxicity. Under the conditions used, iodide was effective at concentrations down to 10^{-5} M and bromide to 10^{-4} M. Chloride was ineffective at 0.1 M. Damage to EPO-coated tumor cells was evident within 5 min of incubation with H_2O_2 and iodide, and reached a maximum at 30 min, under the conditions used in Table II.

Protein inhibits the toxicity of the free peroxidase-H_2O_2-halide system. However, cytotoxicity mediated by cell-bound EPO was markedly less sensitive to inhibition by protein. This is shown in Table III. When free EPO was used, human serum albumin inhibited toxicity partially at a concentration of 4 mg/100 ml and completely at 40 mg/100 ml. In contrast, an albumin concentration of 8 g/100 ml was required for inhibition when EPO was bound to the target cell surface. Similarly, 0.05% serum partially and 0.2% serum completely inhibited toxicity with free EPO, whereas with cell-bound EPO, 30% serum caused only partial inhibition, and inhibition was incomplete at the highest serum concentration employed (40%).

We next undertook studies with three additional tumor cell lines (TLX9 lymphoma, P815 mastocytoma, and NK lymphoma), which vary widely in their susceptibility to lysis by H_2O_2 (TLX9 > P815 > NK) (30), and have been used in earlier studies of the role of H_2O_2 in macrophage-mediated cytotoxicity (3, 5, 30). To mimic the manner in which H_2O_2 is delivered by macrophages, we added glucose oxidase (GO) plus glucose, thereby generating a flux of H_2O_2. We suspended the tumor cells in a complex, serum-containing medium at pH 7.4, without additional iodide or bromide.

The tumor cells were exposed to EPO and then washed to remove unbound enzyme. Such cells showed no signs of toxicity, compared with cells without surface-bound EPO, as judged by exclusion of trypan blue or retention of ^{51}Cr (Table IV).

TABLE I

Toxicity of Tumor Cell-bound EPO*

Supplements	Tumor cell cytotoxicity (percent ^{51}Cr release)	$P\ddagger$	$P\S$
None	10.9 ± 1.4 (3)‖		
LSTRA-EPO + H$_2$O$_2$ + iodide $(10^{-4}$ M)	66.2 ± 1.6 (3)		<0.001
LSTRA-EPO + H$_2$O$_2$ + iodide $(10^{-5}$ M)	60.5 ± 0.9 (6)		<0.001
Iodide omitted	12.0 ± 1.8 (3)	<0.001	
H$_2$O$_2$ omitted	11.9 ± 1.6 (3)	<0.001	
EPO omitted	6.9 ± 0.6 (3)	<0.001	
Azide $(10^{-3}$ M) added	10.8 ± 0.5 (3)	<0.001	
Azide $(10^{-4}$ M) added	10.4 ± 0.7 (3)	<0.001	
Azide $(10^{-5}$ M) added	11.7 ± 0.4 (3)	<0.001	
Azide $(10^{-6}$ M) added	54.7 ± 1.9 (3)	<0.02	
Cyanide $(10^{-3}$ M) added	11.8 ± 1.1 (3)	<0.001	
Cyanide $(10^{-4}$ M) added	14.3 ± 2.2 (3)	<0.001	
Cyanide $(10^{-5}$ M) added	37.6 ± 6.3 (3)	<0.002	
Cyanide $(10^{-6}$ M) added	59.9 ± 2.9 (3)	NS¶	
Aminotriazole $(10^{-3}$ M) added	12.9 ± 1.6 (3)	<0.001	
Aminotriazole $(10^{-4}$ M) added	17.0 ± 4.2 (3)	<0.001	
Aminotriazole $(10^{-5}$ M) added	54.1 ± 1.4 (3)	<0.01	
Aminotriazole $(10^{-6}$ M) added	61.1 ± 0.8 (3)	NS	NS
LSTRA-EPO + H$_2$O$_2$ + iodide $(10^{-6}$ M)	18.3 ± 4.7 (3)		NS
LSTRA-EPO + H$_2$O$_2$ + iodide $(10^{-7}$ M)	14.9 ± 4.2 (3)		<0.01
LSTRA-EPO + H$_2$O$_2$ + bromide $(10^{-3}$ M)	65.0 ± 9.6 (3)		<0.05
LSTRA-EPO + H$_2$O$_2$ + bromide $(10^{-4}$ M)	55.4 ± 12.0 (3)		NS
LSTRA-EPO + H$_2$O$_2$ + bromide $(10^{-5}$ M)	33.2 ± 13.4 (3)		NS
LSTRA-EPO + H$_2$O$_2$ + bromide $(10^{-6}$ M)	17.3 ± 5.3 (3)		NS
LSTRA-EPO + H$_2$O$_2$ + chloride (0.1 M)	13.9 ± 3.1 (3)		

* The reaction mixture contained 3×10^{-2} M sodium phosphate buffer pH 7.0, 1.5×10^{-3} M MgSO$_4$, 1.5×10^{-3} M KH$_2$PO$_4$, 6.7×10^{-2} M Na$_2$SO$_4$, 0.005% gelatin, and the supplements where indicated as follows: 10^5 ^{51}Cr LSTRA tumor cells with bound EPO (LSTRA-EPO); 10^{-5} M H$_2$O$_2$, and iodide, bromide, chloride, azide, cyanide, or aminotriazole (sodium salts) at the concentrations indicated. In tubes with "EPO omitted", LSTRA cells were used without preincubation with EPO, and when 0.1 M NaCl was used, the 6.7×10^{-2} M Na$_2$SO$_4$ was deleted. Incubation 30 min.
‡ P value for the difference from the complete system (LSTRA-EPO + H$_2$O$_2$ + 10^{-5} M iodide).
§ P value for the difference from the standard salt solution alone (None).
‖ Mean $\pm$ SE of (n) experiments.
¶ Not significant.

TABLE II

Kinetics of Tumor Cell Cytotoxicity

Time	Tumor cell cytotoxicity (percent ^{51}Cr release)	
	Control	LSTRA-EPO + H$_2$O$_2$ + I$^-$
min		
5	10.9	42.5
15	12.8	62.1
30	16.1	70.2
60	16.0	70.8

The reaction mixture was as described in Table I with either LSTRA-EPO alone (control) or LSTRA-EPO + H$_2$O$_2$ + I$^-$ $(10^{-4}$ M). Incubation was for the period indicated.

TABLE III

Effect of Albumin or Serum on the Cytotoxic Activity of the Free or Tumor Cell-bound EPO System *

Albumin or serum	Tumor cell cytotoxicity (percent ^{51}Cr Release)							
	EPO				LSTRA-EPO			
	Control	Complete system	P‡	P§	Control	Complete system	P‡	P§
mg/100 ml								
None								
Albumin 2	10.7 ± 1.1 (6) ‖	62.2 ± 2.4 (6)	<0.001		17.6 ± 3.1 (4)	65.0 ± 3.2 (4)	<0.001	
4	10.5 ± 0.6 (3)	54.7 ± 5.7 (3)	<0.002	NS¶				
10	7.5 ± 0.9 (3)	47.4 ± 6.3 (3)	<0.01	<0.05				
20	8.2 ± 1.1 (3)	35.9 ± 7.4 (3)	<0.05	<0.01				
40	7.6 ± 0.6 (4)	16.2 ± 2.9 (4)	<0.05	<0.001				
200	8.0 ± 0.3 (3)	8.9 ± 0.6 (3)	NS	<0.001				
2,000	7.9 ± 0.9 (3)	6.6 ± 0.7 (3)	NS	<0.001	11.4 ± 2.2 (4)	65.6 ± 3.2 (4)	<0.001	NS
4,000					10.4 ± 1.5 (4)	51.3 ± 7.5 (4)	<0.002	NS
8,000					9.4 ± 1.4 (4)	41.5 ± 9.3 (4)	<0.02	NS
					10.4 ± 2.4 (3)	24.9 ± 11.2 (3)	NS	<0.02
Serum (%) 0.02	10.9 ± 0.5 (3)	55.2 ± 1.2 (3)	<0.001	NS				
0.05	11.2 ± 0.4 (3)	43.0 ± 1.6 (3)	<0.001	<0.002				
0.1	9.3 ± 2.3 (3)	29.4 ± 6.7 (3)	<0.05	<0.001				
0.2	8.9 ± 0.8 (3)	11.9 ± 3.2 (3)	NS	<0.001				
1	7.7 ± 1.2 (3)	9.3 ± 1.0 (3)	NS	<0.001				
10	11.1 ± 2.0 (2)	8.5 ± 1.6 (3)	NS	<0.001	11.4 ± 1.6 (4)	65.7 ± 2.4 (4)	<0.001	NS
20					11.6 ± 1.3 (4)	59.1 ± 4.2 (4)	<0.001	NS
30					11.6 ± 1.4 (4)	49.9 ± 3.2 (4)	<0.001	<0.02
40					10.9 ± 2.4 (3)	40.4 ± 0.9 (3)	<0.001	<0.002

* The reaction mixture was as described in Table I with either LSTRA cells and free EPO ("EPO") or LSTRA with surface-bound EPO ("LSTRA-EPO"). Control tubes did not contain any other components of the peroxidase system whereas the complete EPO system contained 224 mU EPO, 10^{-5} M H_2O_2, and 10^{-4} M iodide. Human serum albumin or pooled human serum was added at the concentrations indicated.

‡ P value for the difference between the control and complete system.

§ P value for the difference between the complete system with albumin or serum and the complete system without albumin or serum.

‖ Mean ± SE of (*n*) experiments.

¶ Not significant.

TABLE IV

Lack of Toxicity of EPO Alone *

Tumor	Experiments	Percent cells excluding trypan blue		Percent spontaneous release of ^{51}Cr	
		EPO−	EPO+	EPO−	EPO+
TLX9	14	97.7 ± 0.4‡	94.6 ± 1.0	7.6 ± 0.4	10.9 ± 1.0
NK	7	93.6 ± 2.0	93.8 ± 3.7	12.0 ± 0.7	12.4 ± 0.8
P815	3	98.9 ± 0.6	96.8 ± 0.9	8.6 ± 2.5	8.6 ± 2.3

* ^{51}Cr-labeled control and EPO-coated tumor cells were assessed for viability by trypan blue exclusion immediately after cell preparation and by ^{51}Cr-release 4.5 h later.

‡ Means ± SEM for the number of experiments shown.

Moreover, mice injected with 10^6 EPO-coated P388 cells died at 10.7 ± 0.4 d, which was no later than mice injected with control P388 cells (11.4 ± 0.7 d, seven mice per group). However, when exposed to GO plus glucose, EPO-coated TLX9 lymphoma cells were 15 times more sensitive and NK lymphoma cells 76 times more sensitive to lysis by H_2O_2 than were control, uncoated tumor cells, as judged by the flux of H_2O_2 required for 50% specific lysis (Fig. 1).

EPO alone did not affect the amount of GO necessary to attain 50% lysis of P815 mastocytoma cells (Table V). However, BCNU inactivates glutathione reductase in
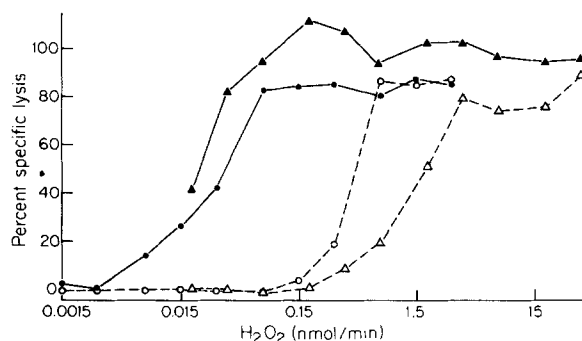
Percent specific lysis

100
80
60
40
20
0

0.0015   0.015   0.15   1.5   15

H_2O_2 (nmol/min)

FIG. 1. Sensitivity of lymphoma cells to lysis by enzymatically generated H_2O_2 with or without prior exposure to EPO. The reaction mixture contained 4 × 10^4 ^{51}Cr-labeled TLX9 (●, ○) or NK (▲, △) lymphoma cells with (●, ▲) or without (○, △) bound EPO in 0.2 ml of MEM-5% HS with 5.5 mM glucose (TLX9) or MEM-0.25% HS with 40 mM glucose (NK). A range of concentrations of GO in 0.02 ml of normal saline was added to individual wells, and ^{51}Cr release determined after 4.5 h of incubation. Glucose was at a higher molarity with NK cells so that higher concentrations of GO could be tested without depleting the medium of glucose. The rate of H_2O_2 generation by GO was measured in Krebs-Ringer phosphate buffer with 5.5 mM or 40 mM glucose, using the scopoletin method, and is indicated on a logarithmic scale on the abscissa. Data points are means of triplicates, for which SEM averaged 2.6 ± 0.5%. Spontaneous release of ^{51}Cr in the absence of GO was: 7.5% (TLX9, EPO−); 7.0% (TLX9, EPO+); 13.0% (NK, EPO−); 10.8% (NK, EPO+). NK lymphoma cells were more sensitive to GO than in a previous study (30) presumably because the serum concentration was lower.

TABLE V

Synergy Between EPO and BCNU in Augmenting Peroxide-Mediated Lysis of P815 Cells*

Experiment	LD50 for un-treated cells	Ratio of LD50 of untreated/treated cells		
		EPO	BCNU	EPO + BCNU
1	0.50	0.93	4.66	13.59
2	0.96	1.10	4.29	11.06

* The reaction mixture was as described in Fig. 1 except that 2 × 10^4 P815 cells in 0.2 ml of MEM-5% HS containing 5.5 mM glucose were used. The cells were either untreated, coated with EPO, incubated with 100 µg/ml of BCNU for 10 min, or both coated with EPO and treated with BCNU. The nmol of H_2O_2/min generated by the amount of glucose oxidase needed to produce 50% specific ^{51}Cr release in 4.5 h (LD50) was determined.

P815 cells and sensitizes them to peroxide-mediated lysis (30). As shown in Table V, the sensitivity of P815 cells to H_2O_2 was increased four- to fivefold by prior exposure to BCNU. The sensitivity of BCNU-treated P815 cells to H_2O_2 was further increased 2.8-fold by surface-bound EPO.

Augmentation of PMA-triggered, Macrophage-mediated Cytolysis by EPO. We next tested the effect of EPO on cytolysis caused by H_2O_2 secreted by macrophages in response to PMA (4). EPO-coated tumor cells were much more readily lysed by PMA-triggered, bacillus Calmette-Guérin (BCG)-elicited macrophages than were native tumor cells (Table VI). With the relatively peroxide-resistant NK lymphoma (30), no macrophage-mediated lysis of tumor cells was observed without EPO, whereas 100% lysis was attained when PMA-triggered, BCG-elicited macrophages were incubated with EPO-coated NK lymphoma cells at effector/target (E/T) ratios of 5:1 to 45:1 (Table

VI, Fig. 2). Similarly, in one experiment, EPO on the surface of NK lymphoma cells greatly increased their sensitivity to lysis by PMA-triggered, casein-induced macrophages. Resident macrophages, however, did not lyse control or EPO-coated NK targets under our experimental conditions (Table VI).

With TLX9 lymphoma cells, the partial lysis observed when uncoated tumor cells were exposed to PMA-triggered, BCG-elicited peritoneal cells was considerably increased when the target cells were coated with EPO (Table VI, Fig. 3). Comparison of E/T ratios at 50% lysis (0.3 for coated and 9.6 for uncoated TLX9) suggested that PMA-stimulated, BCG-elicited peritoneal cells were ~32 times more efficient at lysing EPO-coated TLX9 than native TLX9 (Fig. 3). Even peritoneal cells elicited with thioglycollate medium lysed EPO-coated targets in the presence of PMA. Resident peritoneal cells did so in one of four experiments (Fig. 3).

Spontaneous Macrophage-mediated Cytolysis of EPO-coated Tumor Cells. In contrast to uncoated target cells, EPO-coated NK and TLX9 lymphoma cells were lysed to a substantial extent by BCG-elicited peritoneal cells in the absence of PMA (Table VI and Figs. 2 and 4). Caseinate-elicited cells were also effective in one experiment with NK lymphoma cells (Table VI).

TABLE VI

Augmentation of PMA-induced and Spontaneous Cytolysis by EPO

Target cells*	Effector cells‡	PMA-induced lysis§			Spontaneous lysis‖		
		Number of experiments	EPO−	EPO+	Number of experiments	EPO−	EPO+
NK	BCG¶	3	10.6 ± 4.4**	102.3 ± 5.3	4	3.1 ± 0.9	61.4 ± 5.7
	BCG-FH‡‡	1	4.7	96.7	3	7.5± 2.8	31.5 ± 6.5
	Casein§§	1	0.3	80.5	1	−1.1	29.9
	Res‖‖	2	−1.3 ± 0.1	−0.5 ± 1.2	2	−0.9 ± 0.1	0.8 ± 0.6
	PMN¶¶	3	61.1 ± 7.7	87.2 ± 10.5	3	−2.0 ± 0.6	13.8 ± 7.1
TLX9	BCG	8	33.3 ± 9.2	84.9 ± 7.6	9	9.1 ± 1.6	48.4 ± 8.3
	Casein	1	−0.3	50.0	1	−0.1	16.8
	Thio¶***	2	7.4± 5.6	71.3 ± 8.6	2	0.3 ± 0.3	5.3 ± 2.9
	Res	4	0.7± 0.4	13.5 ± 9.2	5	0.2 ± 0.4	0.1 ± 0.4
	PMN	4	40.6± 20.5	77.9 ± 4.5	8	1.1 ± 0.4	13.3 ± 4.9

* 2×10^4.

‡ 3×10^5, except two experiments with Res cells at 8×10^5.

§ Specific release of ^{51}Cr after 4.5 h with effector cells in the presence of 100 ng/ml PMA in 0.033% DMSO.

‖ Specific release of ^{51}Cr after 4.5 h with effector cells in the presence of 0.033% DMSO.

¶ Peritoneal cells from mice injected intraperitoneally 10 d to 6 wk earlier with 7×10^6 viable BCG, averaging 45.5 ± 2.2% macrophages and 11.5 ± 1.4% granulocytes.

** Mean ± SEM for the number of experiments indicated.

‡‡ BCG peritoneal cells depleted of granulocytes on Ficoll-Hypaque, averaging 59.6 ± 5.8% macrophages and 1.7 ± 0.2% granulocytes.

§§ Peritoneal cells from mice injected intraperitoneally with 1 ml of 6% sodium caseinate 5 d earlier, averaging 62.1 ± 1.6% macrophages and 8.6 ± 1.8% granulocytes.

‖‖ Peritoneal cells from untreated mice, averaging 52.3 ± 6.4% macrophages and 2.0 ± 0.8% granulocytes.

¶¶ Peritoneal cells from mice injected intraperitoneally 16–20 h earlier with 1 ml 6% sodium caseinate (six experiments), 1 ml of 10% thioglycollate medium (one experiment), or 1 ml of 3% proteose peptone (two experiments), averaging 38.0 ± 2.7% macrophages and 55.8 ± 3.5% granulocytes.

*** Peritoneal cells from mice injected intraperitoneally with 1 ml of 10% thioglycollate medium 5 d earlier, containing 69.2 ± 12.4% macrophages and 24.9 ± 10.8% granulocytes.
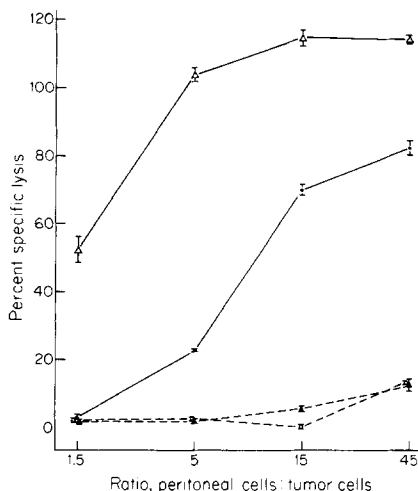
FIG. 2.   Effect of E/T cell ratio. ●, ○ show spontaneous lysis, and ▲, △ show PMA-induced lysis of 2 × 10⁴ control (---) or EPO-coated (—) NK lymphoma cells by BCG-elicited peritoneal cells (see Table VI) in 4.5 h. The number of peritoneal cells was varied to give the peritoneal cell/tumor cell ratios indicated. Means ± SE of triplicate wells are shown.
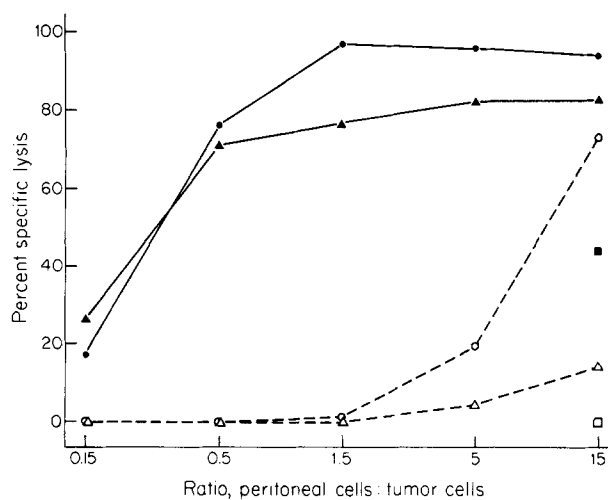

FIG. 3.   Lysis of 2 × 10⁴ ⁵¹Cr-labeled control (○, △, □) and EPO-coated (●, ▲, ■) TLX9 cells by various peritoneal cells in the presence of 100 ng/ml PMA. The peritoneal cells were from untreated mice (Res; ■, □), from mice injected intraperitoneally 5 d earlier with 1 ml of 10% thioglycollate medium (Thio; ▲, △), or from mice injected intraperitoneally 16 d earlier with 7 × 10⁶ viable BCG (BCG; ●, ○), and their number was varied to give the peritoneal/tumor cell ratios shown. Points are means of triplicates, for which SEM averaged 1.5 ± 0.2%.

Peritoneal exudates enriched in granulocytes were collected 16–20 hours after intraperitoneal injection of sodium caseinate, thioglycollate medium, or proteose-peptone. Such granulocyte-rich exudates had little spontaneous cytolytic activity against EPO-coated targets, even at high E/T ratios (Table VI). In contrast, BCG-elicited peritoneal cells, which had been depleted almost completely of granulocytes
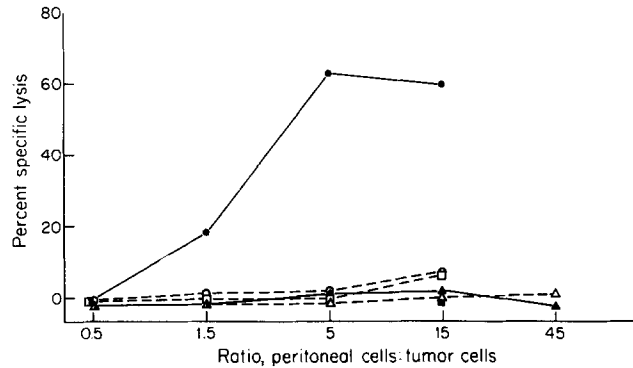
FIG. 4.   Lysis of control (O, △, □) and EPO-coated TLX9 lymphoma cells (●, ▲, ■) by various peritoneal cells in the absence of PMA. Symbols and incubation conditions are as in the legend to Fig. 3. Points are means of triplicates, for which SEM averaged 1.3 ± 0.3%.

on Ficoll-Hypaque, were still cytolytic (Table VI). These results suggested that activated macrophages, rather than granulocytes, mediated spontaneous cytolysis.

BCG-elicited peritoneal cells spontaneously lysed 50% of EPO-coated TLX9 cells in 4.5 h at a peritoneal cell/tumor cell ratio of 3.6, corresponding to a macrophage/tumor cell ratio of 1.5 (Fig. 4). In contrast, at E/T ratios as high as 15:1 to 45:1, resident peritoneal cells and cells elicited with thioglycollate medium remained ineffective (Table VI and Fig. 4). If TLX9 cells were not exposed to EPO, then BCG-activated macrophages caused <10% spontaneous lysis under the conditions tested (Fig. 4).

In one experiment with P815 mastocytoma, BCG-elicited cells released 33% of the ^{51}Cr from the tumor cells in 4.5 h at an E/T ratio of 4.6, when the tumor cells were first exposed to both EPO and BCNU as described in Table V. When either EPO or BCNU was omitted, lysis was not observed.

The spontaneous lysis of EPO-coated tumor cells by BCG-activated macrophages reached 50% of its maximum value in ~1 h, and approached a plateau at ~3 h (Fig. 5). In contrast, there was almost no lysis of control tumor cells by 8 h. Resident peritoneal cells failed to lyse EPO-coated tumor cells spontaneously even after 20 h (data not shown).

In a dose-response experiment, spontaneous lysis of TLX9 cells by BCG-elicited peritoneal cells increased from 5% for control TLX9 cells to a maximum of 89% for TLX9 cells incubated with 0.6 U of EPO and then washed. Half-maximal lysis followed exposure to 0.3 U EPO. To determine how much of the EPO bound to the tumor cells, peroxidatic activity was measured immediately after adding the enzyme to the tumor cell suspension; after a 10-min incubation under standard conditions (see Materials and Methods), the cells were washed four times by centrifugation, and peroxidatic activity was determined again. Both the initial and final assays were done in the presence of 0.04% Triton X100. In four such experiments (two each with TLX9 and NK lymphomas), 22 ± 5% of the enzymatic activity of the added EPO remained bound to the tumor cells.

Lysis of EPO-coated tumor cells by macrophages has been called spontaneous because no additional agent such as PMA was required. However, it was possible that EPO itself, or EPO-coated tumor cells, might act like PMA and stimulate a burst of
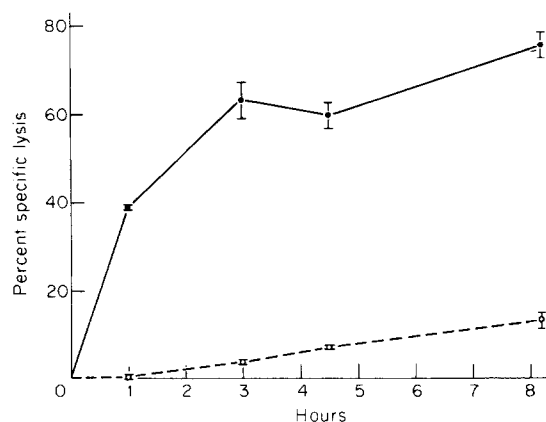
FIG. 5.   Time course of spontaneous lysis of 2×10^4 ^{51}Cr-labeled control (- - -) or EPO-coated (—) TLX9 lymphoma cells by 3×10^5 BCG-elicited peritoneal cells. Means ± SEM for triplicates are indicated.

TABLE VII

EPO-coated Tumor Cells Do Not Trigger Lysis of Adjacent Uncoated Targets

^{51}Cr-labeled TLX9 cells*	Unlabeled TLX9 cells*	Percent Specific Lysis‡	
		PMA absent§	PMA present‖
EPO+	None	58 ± 1¶	91 ± 3
EPO+	EPO−	53 ± 2	97 ± 6
EPO+	EPO+	48 ± 3	88 ± 2
EPO−	None	11 ± 0	67 ± 4
EPO−	EPO−	8 ± 1	61 ± 2
EPO−	EPO+	9 ± 1	71 ± 1

* 2×10^4 cells.
‡ After 4.5 h incubation with 3×10^5 peritoneal cells from BCG-treated mice.
§ DMSO added, 0.033%.
‖ 100 ng/ml in DMSO (final DMSO concentration, 0.033%).
¶ Means ± SEM for triplicate cultures.

H_2O_2 secretion by activated macrophages (2). If this were the case, then non-EPO-coated, peroxide-sensitive tumor cells present in the same culture should be lysed as "innocent bystanders" by the interaction of EPO-coated tumor cells with macrophages. As shown in Table VII, no such lysis was observed, although the bystander cells were readily lysed after addition of PMA. Finally, EPO itself elicited no detectable H_2O_2 release from BCG-activated macrophages, as detected by the scopoletin assay (data not shown).

Dependence of Cytolysis on H_2O_2. In six experiments, catalase inhibited the spontaneous lysis of EPO-coated TLX9 or NK lymphoma cells by BCG-elicited peritoneal cells by an average of 98% (Table VIII). Catalase which had been inactivated by heating had little or no inhibitory effect (Table VIII). 23 U of catalase/ml inhibited cytolysis by 50% (Fig. 6 B). Results were similar with PMA-induced cytolysis of EPO-coated targets (e.g., Fig. 7). Thus, both PMA-induced and spontaneous cytolysis appeared to depend on H_2O_2.

Dependence of Cytolysis on the Enzymatic Action of EPO. Cationic proteins from the

TABLE VIII

Inhibition of Cytolysis by Catalase

Experi-ment	Target*	Catalase	Lysis with-out cata-lase‡	Percent inhi-bition with catalase§	Percent inhi-bition with boiled cata-lase§
		U/ml			
1	TLX9	3,000	56.3	102	ND ‖
2	TLX9	3,000	59.5	97.5	ND
3	TLX9	1,000	48.0	103	ND
4	NK	300	68.9	97.4	11.4
5	NK	300	29.9	91.0	0.0
6	TLX9	30	24.7	99.6	3.2

* 2×10^4 EPO-coated tumor cells.
‡ Percent specific lysis after 4.5 h incubation with 3×10^5 peritoneal cells from mice injected with BCG or sodium caseinate.
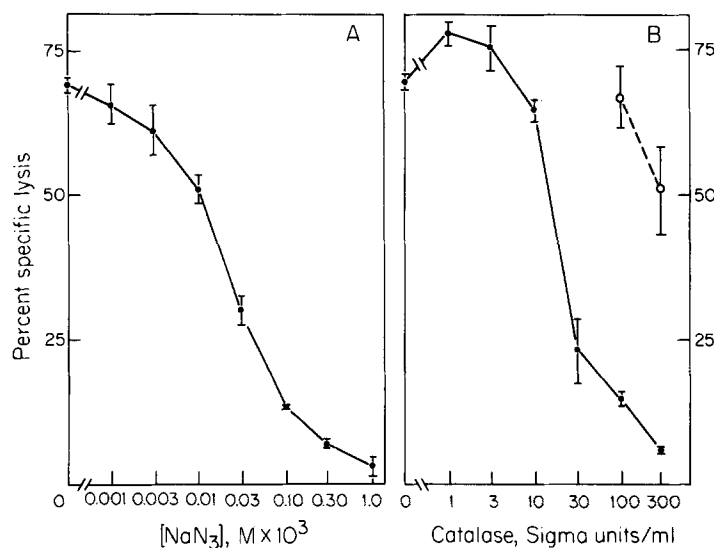§ Calculated as described in Materials and Methods.
‖ Not done.

FIG. 6. Effect of sodium azide (A) and catalase (B) on spontaneous lysis of 2×10^4 ^{51}Cr-labeled, EPO-coated NK lymphoma cells by 3×10^5 BCG-elicited peritoneal cells in 4.5 h. In B, (O) indicate results with heat-inactivated catalase. Means ± SEM for triplicates are shown.

granules of eosinophils (31) or neutrophils (32) may be tumoricidal independent of peroxidase activity. EPO, although a cationic protein, did not seem to exert its toxicity through this property under our conditions, because EPO was nontoxic in the absence of H_2O_2 (Table IV). That EPO was functioning enzymatically in promoting cytolysis was further suggested by two additional approaches.

First, the peroxidase inhibitor sodium azide inhibited spontaneous lysis of EPO-coated TLX9 or NK lymphoma cells by BCG-activated macrophages by a mean of 102% in four experiments (Table IX). Azide at 2.6×10^{-5} M afforded 50% inhibition (Fig. 6A). Sodium azide would not be expected to affect nonenzymatic functions of EPO related to its cationic nature. Second, we exposed NK lymphoma cells to protamine sulfate, a cationic protein (33) lacking in peroxidase activity. Preliminary
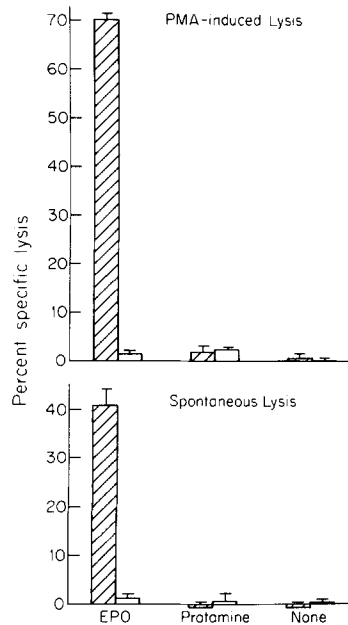
Fig. 7.   Comparison of untreated, protamine-treated, or EPO-treated NK lymphoma cells for their susceptibility to PMA-induced lysis (upper panel) or spontaneous lysis (lower panel) by 3×10^5 BCG-elicited peritoneal cells in 4.5 h. ^{51}Cr release was measured from 2×10^4 NK lymphoma cells incubated in the presence of 300 U/ml of native catalase (□) or heat-inactivated catalase (▨). Means ± SEM for triplicates are indicated. Values for percent spontaneous release with native and boiled catalase were: 8.5, 9.2 (untreated NK cells); 9.8, 9.3 (protamine-treated NK cells, 0.06 mg/ml for 10 min); 9.4, 10.8 (EPO-treated NK cells).

TABLE IX

Inhibition of Cytolysis by Azide

Experi-ment	Target*	NaN₃	Lysis without azide‡	Percent inhibi-tion with azide§
		mM		
1	TLX9	1.0	56.3	105
2	TLX9	1.0	59.5	106
3	NK	0.3	68.9	95.8
4	TLX9	0.03	48.0	99.8

For explanation of notes, see Table VIII.

experiments demonstrated that protamine sulfate alone was toxic to NK lymphoma cells (data not shown). The highest concentration of protamine sulfate that did not itself elevate the spontaneous release of ^{51}Cr from the tumor cells was 0.06 mg/ml. NK lymphoma cells exposed to this concentration of protamine sulfate were unharmed by BCG-activated macrophages, with or without PMA, whereas the tumor cells exposed to EPO (<0.02 mg/ml) were extensively lysed by the macrophages (Fig. 7). As before, lysis was abolished by catalase (Fig. 7).

Discussion

Many reports have documented that myeloperoxidase, lactoperoxidase, or EPO can enhance the lysis of tumor cells by H_2O_2 and halide, whether the H_2O_2 is

preformed, generated by enzymes *in situ*, or released by leukocytes stimulated with particles, lectins, or phorbol esters (25, 29, 34–40). The work reported here extends such observations in two ways.

First, EPO catalyzed the lysis of tumor cells by H_2O_2 released spontaneously from activated macrophages in the absence of exogenous triggering agents. This constitutes a new, unusually rapid, and potent form of spontaneous macrophage-mediated cytolysis. Second, this peroxidase-dependent cytotoxicity occurred in a complex, serum-containing tissue culture medium without further addition of halides. The medium was physiologic, in that it could support the replication of TLX9, P815, and P388 tumor cells at approximately the same rate as in the peritoneal cavity (C. Nathan, unpublished observations). When peroxidases are in solution, their cytotoxic effects are readily inhibited by serum proteins, which compete with cells for the toxic products of the peroxidase system. In contrast, EPO, which is highly cationic (pI > 11) (21), bound to the surface of tumor cells, where it efficiently promoted peroxide-dependent cytotoxicity, despite the presence in the medium of competing compounds such as serum proteins.

Cell-bound EPO sensitized each of four different types of tumor cells to H_2O_2. For three of these, we quantified the degree of sensitization to enzymatically generated H_2O_2. TLX9 lymphoma cells, which are relatively sensitive to H_2O_2 (30), became ~15 times more sensitive after exposure to EPO. NK lymphoma cells, which are comparatively resistant to H_2O_2 (30), became up to 76-fold more susceptible after treatment with EPO. As a consequence, there were fluxes of H_2O_2 that lysed none of the control tumor cells, but essentially all of the EPO-coated cells. On the other hand, the peroxide sensitivity of P815 mastocytoma cells, which is normally intermediate between TLX9 and NK cells, did not appear to be affected by EPO alone. However, when EPO-treated P815 cells were also exposed for 10 min to BCNU to inactivate glutathione reductase, an enzyme involved in H_2O_2 catabolism (30), they became 13 times more sensitive to H_2O_2 than cells treated with neither EPO nor BCNU (2.8 times more sensitive than cells treated with BCNU alone). The basis for this variation in response to EPO among different tumors is unknown, but could relate to differences in binding of the enzyme.

The spontaneous lysis of EPO-coated tumor cells by activated macrophages was rapid (50% at 2 h at a macrophage/tumor cell ratio of 6:1). In contrast, control target cells were almost completely spared during an 8-h incubation with activated macrophages. In assays lasting 4.5 h, macrophage/tumor cell ratios of 1.5:1 to 4.6:1 produced 50% spontaneous lysis of EPO-coated targets. Macrophages activated by injection of mice with either BCG or sodium caseinate were able to lyse EPO-coated tumor cells spontaneously. However, resident peritoneal macrophages or macrophages elicited with thioglycollate medium were ineffective, and peritoneal granulocytes showed marginal cytolytic activity only at high E/T ratios. Spontaneous lysis of EPO-coated tumor cells by activated macrophages was inhibited completely by catalase, with 50% inhibition by 23 U/ml, but not by boiled catalase. Thus, spontaneous cytolysis appeared to require H_2O_2.

We attributed spontaneous cytolysis in this setting to macrophages, for the following reasons. A low level of spontaneous secretion of H_2O_2 by activated macrophages has been measured biochemically (2), and is consistent with the known elevation of hexose monophosphate shunt activity in activated macrophages at rest (41–45). In contrast,

secretion of H_2O_2 by BCG-elicited peritoneal lymphocytes could not be detected (2). Very low levels of spontaneous cytolysis of EPO-coated tumor cells resulted from incubation with large numbers of peritoneal granulocytes. However, granulocyte-depleted populations, consisting almost exclusively of macrophages and lymphocytes, remained spontaneously cytolytic.

We have referred to cytolysis as spontaneous when there was no requirement for exogenous agents known to trigger macrophages. EPO itself did not appear to be such an agent. EPO did not elicit detectable H_2O_2 release from activated macrophages, nor did EPO-coated tumor cells induce macrophages to lyse tumor cells not coated with EPO. However, as with any system of apparently spontaneous cytotoxicity, it is possible that stimuli endogenous to the assay may have elicited a cytotoxic response. Such stimuli could include components of the medium, the surface of the culture vessel, or the tumor cells themselves (46).

EPO is a cationic protein, and like other granulocyte cationic proteins (31, 32), might be tumoricidal by virtue of this property. However, no cytotoxicity was seen with EPO unless H_2O_2 was added. The cytotoxicity of EPO plus H_2O_2 was abolished completely by low concentrations of the peroxidase inhibitor sodium azide (50% inhibition at 2.6×10^{-5} M). This agrees closely with the concentration of azide found previously to inhibit the enzymatic activity of EPO (47). Azide did not inhibit cytotoxicity by reducing macrophage H_2O_2 production (data not shown). When tumor cells were not exposed to EPO, then PMA-triggered cytolysis by macrophages was either unaffected by azide or augmented by it (4). Finally, protamine sulfate, a cationic protein (pI $\approx$ 12) (33), which was cytotoxic in the absence of added H_2O_2 at relatively high concentrations, did not support the expression of spontaneous cytotoxicity by activated macrophages at protein concentrations at least threefold greater than those at which EPO was effective.

It is not known to what extent eosinophils release their granule contents in a tumor bed, or with what efficiency EPO might bind to tumor cells in vivo. However, under our experimental conditions, the EPO contained in $\sim 8 \times 10^4$ eosinophils appeared to be sufficient to sensitize 1×10^6 TLX9 lymphoma cells to undergo 89% specific lysis in the presence of 8×10^6 BCG-activated macrophages. By this estimate, the peroxidase in 1 eosinophil could augment maximally the spontaneous in vitro cytolytic capacity of 100 macrophages.

Titrations of the cytolytic activity of peritoneal cells and of glucose oxidase plus glucose suggest that BCG-activated macrophages would produce the observed degree of lysis of EPO-coated tumor cells if the macrophages spontaneously released about 0.26 nmol of H_2O_2 per min. This figure is an order of magnitude higher than the rate of spontaneous H_2O_2 release previously observed with BCG-activated macrophages in suspension (0.02 nmol/min) (2). There are several possible explanations for this apparent discrepancy.

First, the proximity of macrophages to tumor cells may have raised the local concentration of H_2O_2 to a level higher than would be achieved by soluble glucose oxidase with the same rate of H_2O_2 generation. Second, the peritoneal cell preparations may have supplied factors augmenting the efficiency of EPO. Thus, production of acid by macrophages in the vicinity of tumor cells may have lowered the pericellular pH. At the same concentrations of H_2O_2 and EPO, there is far more toxicity to tumor cells at pH 6 than at pH 7 (25). Peritoneal cell preparations may have supplied traces

of a halide or other oxidizable cofactor. Iodide exerts a synergistic effect with chloride in permitting the halide-dependent toxicity of the EPO-H_2O_2 system (25). In addition, the BCG-elicited peritoneal cell populations contained $0.75 \pm 0.25\%$ mast cells (mean $\pm$ SEM, $n = 12$). H_2O_2 triggers mast cell degranulation (38, 48, 49) and mast cell granules markedly enhance the cytotoxicity of EPO for tumor cells (38).

Finally, macrophages secrete additional cytotoxic substances besides H_2O_2 (8–11). One of these, termed cytolytic factor (11), can exert a synergistic effect together with H_2O_2, so that tumor cells are lysed at concentrations of each substance which are not cytolytic separately (50). Thus, whereas H_2O_2 was necessary for spontaneous macrophage-mediated cytolysis of EPO-coated targets, other factors may have increased the efficiency of lysis.

The findings presented here and earlier (50) raise the possibility that spontaneously secreted H_2O_2 may contribute, along with other factors, to the cytotoxicity occurring during more prolonged contact of activated macrophages with tumor cells (51) in the absence of EPO. In summary, an enzyme from eosinophils and an inorganic substrate released spontaneously by activated macrophages together lysed tumor cells that were unharmed by either agent alone. Cytotoxicity was rapid and potent, and occurred readily in a medium with a composition similar to that of extracellular fluid. These features encourage the speculation that macrophages and eosinophils may cooperate in exerting extracellular cytotoxicity when they migrate together into tumor beds, sites of parasitic infestation, or other foci of inflammation.

Summary

Eosinophil peroxidase (EPO), a cationic protein purified from horse blood, adhered to four different types of tumor cells, markedly potentiating their lysis by preformed or enzymatically generated H_2O_2 (up to 76-fold, as assayed in serum-containing tissue culture medium without supplemental halide). Similarly, compared with uncoated tumor cells, EPO-coated tumor cells were up to 32 times more sensitive to lysis when incubated with macrophages or granulocytes whose respiratory burst was triggered by PMA. However, EPO-coated tumor cells were also readily lysed by bacillus Calmette-Guérin-activated macrophages in the absence of exogenous triggering agents. This spontaneous cytolysis was rapid (50% at 2 h) and potent (50% lysis at macrophage/tumor cell ratios of 1.5 to 4.6), and was observed with both a peroxide-sensitive tumor (TLX9) and a peroxide-resistant tumor (NK lymphoma). Under the conditions used, neither EPO alone nor macrophages alone were spontaneously cytolytic. Neither EPO nor EPO-coated tumor cells triggered a detectable increment in H_2O_2 release from macrophages. Nonetheless, spontaneous macrophage-mediated cytolysis of EPO-coated tumor cells was completely inhibitable by catalase (50% inhibition, 23 U/ml), although not by heated catalase, indicating a requirement for H_2O_2. Cytolysis was also completely inhibitable by azide (50% inhibition, 2.6×10^{-5} M), indicating a requirement for enzymatic activity of EPO. Thus, a cytophilic peroxidase from eosinophils and H_2O_2 spontaneously released from activated macrophages interacted synergistically in a physiologic medium to destroy tumor cells.

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